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Inorganic nitrogen availability alters *Eucalyptus grandis* receptivity to the ectomycorrhizal fungus *Pisolithus albus* but not symbiotic nitrogen transfer

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Summary

- Forest trees are able to thrive in nutrient poor soils in part because they obtain growth-limiting nutrients, especially nitrogen (N), through mutualistic symbiosis with ectomycorrhizal (ECM) fungi. Addition of inorganic N into these soils is known to disrupt this mutualism and reduce the diversity of ECM fungi. Despite its ecological impact, the mechanisms governing the observed effects of elevated inorganic N on mycorrhizal communities remain unknown.
- We address this by using a compartmentalized *in vitro* system to independently alter nutrients to each symbiont. Using stable isotopes, we traced the nutrient flux under different nutrient regimes between *Eucalyptus grandis* and its ectomycorrhizal symbiont, *Pisolithus albus*.
- We demonstrate that giving *E. grandis* independent access to N causes a significant reduction in root colonization by *P. albus*. Transcriptional analysis suggests that the observed reduction in colonization may be caused, in part, by altered transcription of microbe perception genes and defence genes. We show that delivery of N to host leaves is not increased by host nutrient deficiency but by fungal nutrient availability.
- Overall, this advances our understanding of the effects of N fertilization on ECM fungi and the factors governing nutrient transfer in the *E. grandis* **-** *P. microcarpus* interaction.

Keywords

Ectomycorrhizal fungi, Nitrogen deposition, Nutrient trading, Stable isotope tracing, Transcriptomic analysis

Introduction

Nutrient cycling and the health of forest ecosystems are affected by the presence of mutualistic ectomycorrhizal (ECM) fungi. Through symbiosis with tree roots, these fungi have key roles in aiding the tree gain access to growth-limiting nutrients such as nitrogen (N) in return for photosynthetically fixed carbon (C) from the tree host. ECM fungi also provide other ecosystem services such as increased sequestration of C and improved soil stability through aggregation (Rillig & Mummey, 2006; Clemmensen *et al*., 2013). While ECM relationships occur most frequently where inorganic N sources within the soil are limited and N is instead found primarily in organic forms (Read *et al*., 2004; Toljander *et al*., 2006; Lin *et al*., 2017), an increasing number of studies have shown that inorganic N enrichment in forest soils through natural causes, pollution or intentional fertilization are leading to a reduction in the level of plant root colonization by ECM fungi and a community shift in soils away from ECM fungi specialized in organic N acquisition to more generalist nitrophilic species and saprotrophs (Lilleskov *et al*., 2002; Parrent *et al*., 2006; Pardo *et al*., 2011; Morrison *et al*., 2016; Corrales *et al*., 2017; Averill *et al*., 2018). Therefore, as inorganic N availability continues to rise in forest soils (Galloway *et al*., 2004; Hietz *et al*., 2011) the negative outcomes to ecosystem services provided by ECM fungi are expected to increase (Pheonix *et al*., 2012; Field *et al*., 2014). However, a mechanism behind these observations is lacking.

One possibility for the observed reduction in ECM communities and colonization of hosts in N-rich environments is that increased nutrient availability alters the supply/demand paradigm of the mutualism, thereby disrupting fungal establishment and persistence on the root system. While nutrient exchange stands as the hallmark of ECM symbiosis, very little is known about what induces the transfer of N from the fungal symbiont to its host (Garcia *et al*.,

2015). There is some evidence from another lineage of mycorrhizal fungi, the arbuscular mycorrhizal (AM) fungi, that exchange of nutrients is based on a reciprocal rewards system, where fungal delivery of nutrients to the plant host is tied to, or “rewarded” with, increased C supply (Kiers *et al*., 2011; Fellbaum *et al*., 2012; Fellbaum *et al*., 2014), though this may be context-dependent (Stonor *et al*., 2014; Walder & van der Heijden, 2015). While little correlation between N transferred by the fungus and C returns from the host has been shown in ECM fungal associations (Corrêa *et al*., 2008; Albarracín *et al*., 2013; Valtanen *et al*., 2014; Hortal *et al*., 2017), soil N levels, particularly the availability of inorganic N, can alter C/N trading dynamics (Treseder, 2004, Albarracín *et al*., 2013; Näsholm *et al*., 2013; Hasselquist & Högberg, 2014). This may disrupt the stability of the mutualism and lead to the observed ecological outcomes. The plant may actively exclude the fungus from accessing its tissues through up-regulation of defence-related pathways in an effort to conserve its own C resources when N availability is high. In arbuscular mycorrhizal symbioses, several studies have shown that host plants actively suppress microbial colonization when nutrients are plentiful (Breuillin *et al*., 2010; Balzergue *et al*., 2011; Nouri *et al*., 2014; Kobae *et al*., 2016), although the mechanism of this effect remains unknown (Kobae *et al*., 2016).

The main difficulty in interpreting the effects of nutrient availability on the symbiosis between plant and fungal partners is that they are typically grown in a common substrate, so nutrients available to one partner are also available to the other. This makes it difficult to determine true mechanistic cause and effect concerning how symbiosis is disrupted. Using a nutrient compartment-based *in vitro* system and stable isotope tracing of C and N, we remove some of these limitations by giving the model ECM fungus *Pisolithus albus* and its host *Eucalyptus grandis* access to independent nutrient

regimes. Specifically, we vary plant access to N and, independently, fungal access to C. Our results from this fully factorial experiment suggest that increased N nutrition to the plant alone systemically induces alterations to plant-encoded microbial perception pathways and increases in chemical defence pathways in a manner that correlates with a reduction in the colonization of roots by the ECM fungus. Further, we show that symbiotic delivery of N from the fungus to the host plant leaves is not dependent on host access to N. Rather, N supply to the host is increased when the fungal partner is given less C. These results add to our knowledge and understanding of nutrient exchange dynamics and the effect of fertilization in this ecologically important relationship.

Materials and Methods

Compartment Setup and Microcosm Design

Tri-compartment 90-mm petri dishes were used to test the effect of substrate nutrition on ECM symbiosis between *P. albus* and *E. grandis* roots. The three compartments were named the 'symbiotic compartment' (SC), the 'plant-only compartment' (PC) and the 'fungus-only compartment' (FC; Fig. **1a**). The compartmental design was accomplished using a divided 90-mm petri dish to separate the SC and PC and by embedding a centrifuge tube screw cap (Edwards Co., cap capacity 1.2 mL) into the SC when media was solidifying to create the FC. Thus, all compartments were separated by solid plastic barriers to completely prevent the diffusion of nutrients from one compartment to another, allowing us to vary nutrients in such a way as to allow only plant or fungal access to a prescribed set of nutrients.

The SC contained 0.1 % glucose solidified with agar. The PC contained half strength Modified Melin- Norkrans (MMN) media with 0.1 % glucose added (Plett *et al.,* 2015). Within this medium, as we were interested in the impact of inorganic N availability, we created either a "high" N condition (10 mM total N with a NH_4:NO_3 at a ratio of 2.2:1 plus 1 x 10^{-3} mM organic N from thiamine) or a "low" N condition without added inorganic N (ammonium nitrate and diammonium phosphate were replaced with sodium hydrogen phosphate to maintain phosphorus balance; 1 x 10^{-3} mM N from thiamine in media). The FC contained full-strength MMN media either with 1.0 % glucose (high fungal C condition) or 0.1 % glucose (low fungal C condition). Within the FC, all inorganic N was enriched in ^{15}N ($^{15}NH_4Cl$; Sigma-Aldrich; $\geq$98 atom % ^{15}N). More detailed information on the media composition of each compartment can be found in Supporting Information Table S1. Plates were made to create all four combinations of high or low nutrients to each partner

to enable a fully factorial experimental design. Agar surfaces were covered with sterile cellophane membranes to prevent root and fungal penetration into the agar.

Fungal and Plant Material

Small squares (0.5 cm x 0.5 cm) of *Pisolithus albus* (isolate SI12; Plett *et al*., 2015) were excised from the leading edge of a one-month old colony growing on full strength MMN media (1.0 % glucose). These were placed in the SC of the test plates, just below the FC nutrient reservoir. They were grown for two weeks at 25^{o}C to allow for growth of the mycelium over the plastic barrier and into the FC nutrient reservoir. *E. grandis* seedlings (lot 20974; CSIRO seed bank), prior to addition to the microcosm, were surface sterilized with 30% hydrogen peroxide for 10 minutes, rinsed three times in sterile water and germinated on 1.0 % agar plates for four weeks post-sterilization followed by four weeks of growth on 1/2 MMN plates (0.1 % glucose). Plates were sealed with Micropore tape to allow gas exchange. Plants were grown in growth chambers (Climatron-1260, Thermoline Scientific) with a daytime temperature of 25^{o}C and night temperature of 18^{o}C and a 16-hour photoperiod (RH: 60 %; light level: 900 μmol/m^2). As we wished to trace carbon movement within our microcosm using stable isotopes, we equipped the growth chamber with Sodasorb scrubbers to remove all CO_2 from the air and replaced it with 400 pm CO_2 depleted in ^{13}C (Aligal 2, Air Liquide Australia). This method depleted natural levels of $^{13}CO_2$ in the air such that plant and fungal samples were sufficiently different in ^{13}C isotopic abundances to trace the fungal C originating from the plant (i.e. carbon with a higher ^{12}C signature would more likely come from plant photosynthate while carbon with a higher ^{13}C signature would come from fungal C sourced from the glucose in the FC medium). The chamber air had an average atom %^{13}C of 1.0844 and the plants were grown in these chambers from seed to the end

of the experiment, giving them an atom %^{13}C ranging from 1.066-1.069 (see Supporting Information Figure S2).

Microcosm Setup and Harvest

Eight-week old seedlings with branched root systems were selected for the study and placed on the tri-compartment test plates with the pre-grown fungus such that half of the root system was in contact with the fungus in the SC, with no roots placed in or near the FC, and the other half of the root system was given access to the PC. Plant-only and fungus-only control plates were also produced. Plates were sealed again with Micropore tape and placed in the growth chambers described above at a 45^{o} angle and rotated slightly so that condensation would not pool at the interface of the SC and PC sections, which would have led to unwanted nutrient mixing. After 48 hours of fungal contact, three biological replicates from each nutrient condition of the experiment were harvested for RNA extraction and analysis. The remaining plants were left for a total of two weeks of contact with the ECM fungus before harvesting.

At harvest, the percentage of root tips colonized by *P. albus* was recorded (number of mycorrhizal root tips/total lateral roots in contact with fungus x 100%). Averages are from a total of 12 replicates per nutrient condition harvested over a series of three independent experiments to ensure reproducibility. Leaves from the plant and the mycelium of the fungus were collected for five biological replicates per nutrient condition as well as three biological replicates of plant-only and fungal-only controls and dried at 40^{o}C overnight. All samples for isotopic analysis originated from the same experiment to reduce labelling biases between experiments. These were ground up, weighed and sent for ^{13}C/^{15}N analysis by an elemental analyser and isotope ratio mass spectrometry (UC Davis Stable Isotope Facility, Davis, California, USA).

Isotopic calculations

Fungal and leaf C/N ratios were calculated based on the ratio of the total mg C and mg N present in the dried samples as determined by stable isotope analysis. Similarly, the N content of the plant leaves was determined by multiplying the ratio of the mg N to mg total sample (as determined from the isotopic data) by the leaf dried biomass. The leaf biomass was measured for each plant after harvest and drying. The percentage of total N as ^{15}N in the fungus was calculated as the ratio of mg ^{15}N to mg total N multiplied by 100 %.

The percentage of N in the plant derived from transfer from the fungus (% NDFT) was calculated according to the formula (He *et al*., 2009; Tomm *et al*., 1994):

$$\%NDFT = \frac{(^{15}N_{Plant} - 0.38)}{(^{15}N_{Fungus} - 0.38)} \times 100\%$$

where $^{15}N_{Plant}$ and $^{15}N_{Fungus}$ are the atom percentages of ^{15}N in the dried plant leaves and fungal mycelium as determined by stable isotope analysis, respectively. The value of 0.38 represents the average background atom percentage of ^{15}N found in our unlabelled experimental controls. It should be noted that the calculated % NDFT refers to the percent contribution of fungal N to the total N pool of the plant leaves, not just that N that was acquired during the two-week plant-fungal contact period.

The quantity of N received by the plant leaves via symbiosis was calculated using % NDFT as above, divided by 100 to express as a fraction, and multiplying it by the N content of the plant leaves in micrograms.

The percentage of C acquired by the fungus via symbiosis was calculated according to the formula:

$$\%C_{symbiosis} = \frac{(^{13}C_{Fungus} - 1.0958)}{(^{13}C_{Plant} - 1.0958)} \times 100\%$$

where $^{13}C_{Fungus}$ and $^{13}C_{Plant}$ are the atom percent ^{13}C values of the dried fungal mycelium and plant leaves respectively, as determined by stable isotope analysis. The value of 1.0958 represents the average atom percent ^{13}C value for control fungi not in contact with a plant. As with the plant calculations, $\%C_{symbiosis}$ represents the percent contribution of plant carbon to the total mycelial C pool, not just that obtained during the period of contact. Estimated micrograms of carbon transferred to the fungal colonies from the plant during contact were calculated using $\%C_{symbiosis}$ (divided by 100 to express as a fraction), and multiplying by the C content of dried mycelium (as determined from the stable isotope data) and an estimated biomass value of 472 μg for high C samples and 247 μg for low C samples. These values were based on the average biomass of control fungi not in contact with plants as it was not possible to accurately record the mass of colonies in contact with plant roots as the two tissue types could not be fully separated.

Nutrient Calculation Statistical Analyses

Two-way ANOVAs (unbalanced, Type III) were used to examine the effect of glucose or nitrogen availability and their interaction on experimental outcomes using the “car” package in R (R Core Team, 2016; Fox & Weisberg, 2011). Normality of the data was tested using the Shapiro-Wilk normality test and homogeneity of variance using the Bartlett’s test in R (R Core Team, 2016). All data conformed to a normalized and homogeneous distribution ($p > 0.05$). Any statistical outliers were removed from the dataset (based on the interquartile range (IQR) method). In all instances, results with a p-value of less than 0.05 were determined to be significant.

RNA extraction and transcriptomic analysis

For 48-hour time point samples, roots in the symbiotic compartment were excised along with any attached fungal mycelium and snap frozen in liquid nitrogen. RNA was extracted with the Qiagen RNeasy plant micro kit according to manufacturer's instructions. RNA for three replicates each of the high N/low C and low N/low C conditions were sequenced at the Joint Genomes Institute (JGI). Plate-based RNA sample prep was performed on the PerkinElmer Sciclone NGS robotic liquid handling system using Illumina's TruSeq Stranded mRNA HT sample prep kit utilizing poly-A selection of mRNA following the protocol outlined by Illumina in their user guide: http://support.illumina.com/sequencing/sequencing_kits/truseq_stranded_mrna_ht_sample_prep_kit.html, and with the following conditions: total RNA starting material was 100 ng per sample and 10 cycles of PCR was used for library amplification. The prepared libraries were quantified using KAPA Biosystem's next-generation sequencing library qPCR kit and run on a Roche LightCycler 480 real-time PCR instrument. The quantified libraries were multiplexed with other libraries, and the pool of libraries was then prepared for sequencing on the Illumina HiSeq sequencing platform utilizing a TruSeq paired-end cluster kit, v4, and Illumina's cBot instrument to generate a clustered flow cell for sequencing. Sequencing of the flow cell was performed on the Illumina HiSeq2500 sequencer using HiSeq TruSeq SBS sequencing kits, v4, following a 2 x 150 indexed run recipe. Raw RNA-Seq reads were filtered and trimmed using the JGI QC pipeline. Using BBDuk (https://sourceforge.net/projects/bbmap/), raw reads were evaluated for artifact sequence by kmer matching (kmer=25), allowing 1 mismatch and detected artifact was trimmed from the 3' end of the reads. RNA spike-in reads, PhiX reads and reads containing any Ns were removed. Quality trimming was performed using the phred trimming method set at Q6. Finally, following trimming, reads under the length threshold were removed (minimum length 25 bases or 1/3 of the original read length - whichever is longer).

Filtered reads from each library were aligned to either the *E. grandis* (Myberg *et al*., 2014; https://phytozome.jgi.doe.gov/pz/portal.html#!info?alias=Org_Egrandis) or *P. albus* SI12 (https://genome.jgi.doe.gov/Pisalb1/Pisalb1.home.html) reference genome using HISAT2 version 2.1.0 (Kim *et al.*, 2015; BAMs/ directory). Only primary hits assigned to the reverse strand were included in the raw gene counts (-s 2 -p --primary options). Features assigned to the forward strand were also tabulated (-s 1 -p --primary options). Strandedness of each library was estimated by calculating the percentage of reverse-assigned fragments to the total assigned fragments (reverse plus forward hits). DESeq2 (version 1.18.1; Love *et al*., 2014) was subsequently used to normalize data and determine which genes were differentially expressed between pairs of conditions. Genes differentially regulated in the plant high N condition as compared to the plant low N condition were considered. Adjusted p-values were calculated by incorporating a Benjamini-Hochberg FDR correction. For *E. grandis*, genes differentially regulated by more than 5-fold from controls were considered ($p < 0.05$) and for *P. albus*, genes differentially regulated by more than 2-fold were considered ($p < 0.05$). Gene annotations, gene ontology (GO) terms and signal peptide probability scores were assigned to each significantly differentially regulated gene based on annotation data from the online *E. grandis* or *P. albus* SI12 genome resources given above.

GO term enrichment analysis was performed using the web-based platform PlantRegMap (plantregmap.cbi.pku.edu.cn/go.php). Significantly up- and down-regulated genes in the *E. grandis* data set were assessed separately and GO terms within the Biological Process aspect were considered as significantly enriched with a threshold p-value of 0.01 based on a Fisher's exact test. Q-values represent the adjusted p-value based on the Benjamini-Hochberg

362 method. Scatterplot visualization of results was performed using
363 the online platform ReviGo (revigo.irb.hr; Supek *et al*., 2011) using
364 default settings for a large size data set.
365
366

Results

Formation of mycorrhizal root tips was driven primarily by plant nutritional needs

The use of a tri-compartmented system to give *E. grandis* independent access to high or low plant available inorganic nitrogen and *P. albus* independent access to high or low glucose was successful (Fig. **1a**). After two weeks of symbiotic establishment between the two organisms, the percentage of mycorrhizal root tips formed in each nutrient combination was determined (Fig. **1b**). For both *E. grandis* and *P. albus,* an increase in resource availability in their respective compartments resulted in reduced root colonization, however this was only significant for the fungus when plant N was high. The lowest percentage of mycorrhizal root tips were formed when the plant had access to high N and the fungus had access to high C, while the greatest percentage of mycorrhizal root tips were formed when both had limited resources. Analysis of variance (ANOVA) demonstrates that N level to the plant was a statistically significant factor in determining the level of root colonization (Table 1).

Plant symbiotic N acquisition was fungal driven

The C/N ratio of *E. grandis* leaves was determined (Fig. **1c**). Plants grown with access to high N had lower C/N ratios in their leaves as compared to those grown without N. Whether or not the plant was associated with *P. albus* did not significantly affect the leaf C/N ratio over the time-course of the experiment. Using stable isotope labelling, we were able to determine that the plant was able to obtain N from the fungus (Fig. **1d**). The amount of plant N acquired via symbiosis (as determined by the abundance of ^{15}N in leaves) was dependent not on the N available to the plant, but on the C available to the fungus (Table 1). That is, a higher quantity of plant N was acquired from the fungus when the fungus had low access to C (Fig.

1d). Plant leaf biomass was not significantly affected by any of the conditions (Supporting Information Figure S1). Plants grown without access to N had significantly less total N in their leaves and thus symbiotically acquired N made up a slightly larger percentage of the total leaf N acquired over the lifetime of the plant, but C availability to the fungus remained the only statistically significant factor in explaining the percentage of plant N derived from transfer as well (%NDFT; Supporting Information Figure S1). Plant N levels and calculations are based on the leaf tissues only, thus we cannot exclude the possibility that there is a treatment effect on N uptake or partitioning between plant shoot and roots.

Fungal acquisition of N was influenced by plant nutritional status

The approximated amount of C transfer from the plant to *P. albus* was not significantly different in any condition suggesting that C and N transfer in this symbiosis is not tightly connected (Fig. **2a**). While resource availability to either plant or fungus were not significant factors in the amount of C transferred (Table 1), fungal colonies with access to low C resources were smaller, and thus the C acquired from the plant made up a larger percentage of the total C acquired over the lifetime of the fungal colony (Supporting Information Figure S2). The percentage of N as ^{15}N for each *P. albus* colony was also considered (Fig. **2b**). As all N supplied to the *P. albus* colony was labelled, a higher percentage of ^{15}N was indicative of greater acquisition of N from the medium for a given fungal colony size. Axenically grown fungal colonies (i.e. without a plant host present) with access to higher levels of glucose acquired a greater percentage of ^{15}N (Fig. **2b**) and were larger than those grown with low levels of glucose (Supporting Information Figure S2). Fungal C/N ratios did not change significantly in any condition (Fig. **2c**). The addition of a plant with high N resources reduced the average percentage of ^{15}N in the fungal colonies. This could be due to lower acquisition from the FC or to loss of N via delivery to the plant.

When the added plant had access to less N resources there was a significant increase in the percentage of ^{15}N in the fungus, no matter how much exogenous C it was provided with. This increase in fungal N is unlikely to be due to reduced transfer to the plant, as this was not significantly altered by the plant N availability (Fig. **1d**), nor did plant N availability affect the amount or percentage of fungal C acquired from the plant. Two-way ANOVA results demonstrate that both fungal C and plant N availability significantly, and independently, affect the ^{15}N levels within the fungal mycelium (Table 1). Thus, low N resources on the part of the plant can result in a greater accumulation of N in the fungus.

N availability altered the transcription of plant genes associated with microbe sensing and sugar metabolism

After 48-hours of contact with *P. albus*, the regulation of *E. grandis* genes in roots from the symbiotic compartment and in contact with the fungus was considered. A comparison of these roots from plants with access to high N versus those from plants without access to N revealed a set of 1,398 differentially regulated genes (760 up-regulated, 638 down-regulated; $p < 0.05$; minimum of 5 times differential regulation; Supporting Information Table S2). This early time point was chosen to consider the differential regulation of genes that may impact the eventual formation of mature mycorrhizal root tips, as our results demonstrated that plant available N had a significant effect on mycorrhizal root tip formation. At this time point, fungal mycelium had begun to wrap around lateral roots and metabolic and transcriptomic responses to contact were already occurring (Wong *et al.*, 2019), but no symbiotic structures were formed. As these roots were in contact with the fungus in the symbiotic compartment, any differentially regulated genes represent a systemic effect of the nutrient status of roots within the plant compartment. GO term enrichment analysis showed a variety of significantly ($p < 0.01$) enriched terms in the area of

biological process (Fig. **3**, Supporting Information Table S3). Particularly, stress response pathways were altered; with an increase in expression of oxidative stress associated genes and a corresponding decrease in hydrogen peroxide catabolic and redox processes. Osmotic stress pathways were also up-regulated. Cell wall polysaccharide associated genes were significantly up-regulated, while lignin catabolism was down-regulated, pointing to a potential strengthening of cell walls. There was also a shift in cell communication and signalling pathways.

185 of the differentially regulated genes (95 up-regulated, 90 down-regulated), representing 13.2 % of all significantly regulated genes, were classified as disease resistance proteins, including leucine-rich repeat (LRR) receptor kinases. Additionally, another 49 membrane bound receptors (20 up-regulated, 29 down-regulated) were differentially regulated. This suggests a substantial shift in the network used by the roots to sense and respond to the surrounding environment and may affect the ability of the plant to perceive and respond to the fungus. Also of interest, 13 galactinol synthase genes, one raffinose synthase and three stachyose synthase genes were significantly up-regulated, suggesting increased flux into the synthesis of raffinose family oligosaccharides.

Plant responses to N availability caused transcriptomic changes in the fungal secretome

When *P. albus* was kept in a consistent nutrient environment but the plant partner was given access to high N, a slight change in the fungal transcriptome is seen 48-hours after contact. Overall, 404 genes were significantly differentially regulated in the fungus (272 up-regulated, 132 down-regulated; $p < 0.05$; minimum of 2 times differential regulation; Supporting Information Table S4). Of these genes, 17.1 % had a predicted secretion domain. Of note, some nutrient transporters were down-regulated, including a nitrate

transporter and a phosphate permease. Two ammonium transporters were also differentially regulated, one up and another down. Genes for secreted proteins involved in host cell wall degradation, including pectin lyases were down-regulated while five heat shock proteins were up regulated. A fungal hydrophobin, important in the establishment of symbiosis (Plett *et al*., 2012), was also down-regulated. Overall the transcriptomic data showed a shift in the production of metabolic proteins, nutrient transporters, a slight increase in stress response (e.g. heat shock proteins) and a change in the fungal secretome.

Discussion

The subject of what drives nutrient exchange between ECM fungi and their hosts is one of great interest within the literature (Müller *et al*., 2007; Garcia *et al*., 2015). While there is little dispute that nutrients are exchanged between the two partners during ECM symbiosis, the factors driving resource exchange are poorly understood. Understanding this is especially important given the finding that inorganic N deposition in forest soils is leading to a reduction of ECM symbiotic associations and communities in forests (Lilleskov *et al*., 2002; Parrent *et al*., 2006; Pardo *et al*., 2011; Morrison *et al.*, 2016; Corrales *et al*., 2017; Averill *et al*., 2018). This effect is not restricted to forests in the Northern Hemisphere; the decline of Eucalyptus forests in Australia has also been linked to the reduction of ECM associations caused, in part, by inorganic N addition (Horton *et al*., 2013). Our results add important information into this subject by determining the effect that N fertilization has on the plant host response to ECM fungi and how increased exogenous C-availability to the fungus affects nutrient transfer in the model interaction between *E. grandis* and *P. albus*. We have shown that at low plant N availability, *E. grandis* was able to promote colonization of its roots, compared to when N was plentiful, where it acted to reduce the number of root tips colonized (Fig. **1b**). The amount of N acquired by *E. grandis* from its symbiont, however, was, in this experiment, independent of the plants' nutritional needs (Fig. **1d**). This means that N flux was not determined by a source/sink system, nor was it based on the level of root colonization by the fungus. Neither was it dependent on C transfer from plant to fungus as this was constant in all conditions. N transfer instead appears to be controlled by the ECM fungus, with the greatest transfer of N occurring when *P. albus* has access to less exogenous C.

The symbiotic transfer of N to a host can be considered as a two-step process where N is first acquired by the ECM fungus from the

environment and then transferred from the fungus to the host. Our results suggest that the first step of this process is influenced by the nutrient status of both the ECM fungus and the plant and that N uptake is dependent on the availability of nutrients in the substrate. *P. albus* acquired more N from the substrate when it was given plentiful C, likely to support the growth of fungal biomass rather than investment in symbiosis as fewer or equivalent numbers of mycorrhizal root tips were formed. This is in contrast to another study using the ECM fungus *Suillus grevillei* where similar high levels of glucose in an *in vitro* system resulted in greater fungal aggression and host, or even non-host, root colonization (Duddridge, 1986). The host nutrient status also appears to influence fungal N uptake: when the plant has access to plentiful N, there was a significant reduction in ^{15}N scavenged by the fungus as compared to when the plant had limited N resources (Fig. **2b**). As the abiotic environment of the fungus had not changed, this decrease in N acquisition could stem from altered resource availability at the plant-fungal interface or via a communication change on the part of the plant. Another study found a dependence on C availability for the decomposition and uptake of soil N by ECM fungi, suggesting that C supply from the plant can influence the uptake of N by the fungus (Rineau *et al*., 2013). While in our experiment there was no difference in net C transfer between conditions, C flux across individual mycorrhizal root tips could vary. Alternatively, even at 48 hours post-contact, prior to the formation of a mycorrhizal root tip, N transporters in the fungus were found to be significantly differentially regulated in response to a plant with high N resources. Thus, a transcriptional change on the part of the plant from altered resource availability may be sensed by the fungus, causing the alteration in its N acquisition strategy. In either case, the reduction in fungal N scavenging seen when the plant has high N resources may in part explain observations showing that N fertilization can cause ECM fungi to halt or slow hyphal growth, have reduced

biomass or reduced competitiveness against other soil organisms (Bidartondo *et al*., 2001; Lilleskov *et al*., 2002; Nilsson & Wallander, 2003; Ekblad *et al*., 2016).

While the nutrient status of both symbiotic partners play a role in the process of fungal N acquisition from the environment, N delivery to the host appears to be primarily under the control of the fungus, based on our results. While fungal acquisition of additional N resources may over a longer term result in more N transfer, over the time frame of our experiment, lack of access to N on the part of the plant did not result in a significant change in N delivery from the fungus. This analysis does not account for the N content of plant roots, and therefore we cannot exclude the possibility that N partitioning or N acquisition from the fungus may be different in these tissues. However, increased exogenous C availability to *P. albus* did result in both a decrease in the amount of N transferred to the plant and a trend to fewer mycorrhizal root tips formed. In experiments using plant shading to alter carbon transfer to ECM fungi, Hasselquist et al. (2016) found a similar result with less N delivery to plant tissues when carbon transfer was high (unshaded). The decrease in N transfer in our experiment was not likely associated with a scarcity of N resources for *P. albus* as colonies with access to high C acquired more N from the substrate and maintained a similar C/N ratio to those colonies with limited access to C. Overall, these results correspond well with previous field-based studies showing that under low N conditions, while trees allocate more C to their roots (Högberg *et al*., 2010; Corrêa *et al*., 2011), this does not appear to correspond to an increase in N gain from the fungus (Corrêa *et al*., 2011; Näsholm *et al*., 2013; Valtenen *et al*., 2014).

In addition to altering the balance of N uptake and transfer between ECM fungi and their hosts, nutrient availability affected the number

of mycorrhizal root tips formed. Transcriptomic analysis of genes differentially regulated in *E. grandis* roots at an early time point in the symbiotic interaction suggests that the addition of N altered how the plant perceived and responded to the presence of *P. albus*. Specifically, we found that there was a large transcriptional reprogramming for a number of disease resistance proteins and membrane bound receptor kinases. Disease resistance proteins, including those containing LRR-RLK (leucine-rich repeat receptor-like protein kinase) domains, make up a large family and are found extensively in the genomes of most plants (Wu *et al*., 2016). Their function is not well understood but they are thought to act as early detector networks of microbial presence and/or effectors and alter signal transduction accordingly to activate plant defence pathways (McHale *et al*., 2006; Smakowska-Luzan *et al*., 2018). Similarly, membrane bound receptor proteins such as lectin receptor kinases are able to sense the presence of microbial elicitors and trigger downstream defence pathways (Singh & Zimmerli, 2013; Tang *et al*., 2017). Thus, the large shift in transcription of these types of genes may signify that the plant has altered how it perceives its external environment and may change its response to the colonizing fungus. Increased expression of galactinol, raffinose and stachyose synthase genes, often up-regulated in response to stress or to defend against a pathogen (Sengupta *et al*., 2015), may result in lower pools of simple carbohydrates available for exchange with a symbiont. Generally mutualistic interactions are characterized by plant cell wall softening and decreased ROS (Plett & Martin, 2017), however, in our data set, N addition resulted in enrichment of genes associated with plant cell wall lignification and increased ROS. Thus, the addition of high levels of inorganic N to a plant causes a systemic transcriptomic shift in tree roots that is overall inhospitable to ectomycorrhizal colonization.

Beyond resource availability in the medium, other factors can affect the level of *E. grandis* root colonization in an *in vitro* system. Elevated levels of CO_2 were shown to reduce the colonization ability of this same isolate of *P. albus*, while in some other related *Pisolithus* isolates, elevated CO_2 increased colonization (Plett *et al*., 2015). It was hypothesized that some *Pisolithus* isolates were more easily able to adapt to or benefit from the plant transcriptional re-programming resulting from the increase in CO_2. Thus, in a similar manner, the effects of N availability on root colonization may be influenced not only by the transcriptomic response of the plant to its nutrient rich environment, but also by the individual fungal susceptibility to that response. In another study involving the interaction of *E. grandis* with the closely related *P. microcarpus*, *E. grandis* was shown to reduce the number of root tips colonized by a less cooperative symbiont (delivering less N for C than its competitor) when a more cooperative competitor was present (Hortal *et al*., 2017). Transcriptomic analysis showed that plant defence genes were up-regulated only in roots in contact with the less cooperative symbiont. While in both the Hortal study (2017) and in our present study, this altered defence signalling was correlated to decreased formation of ECM root tips, it is interesting to note that both C transfer to the fungus and N received in return was unchanged in the earlier study. It would be of interest to explore potential benefits of this defensive reaction on the part of the plant if it does not improve either the costs or benefits of symbiosis.

Overall, here we have significantly expanded our knowledge on how nutrient trading occurs in the interaction between *E. grandis* and *P. albus*. We have shown that plentiful nutrient resources for the plant partner reduced root colonization and that, while the nutrient status of *E. grandis* may influence *P. albus* to scavenge for N, the plant was unable to influence the transfer of that N to its own tissues over the

673 timeline of our experiment. N transfer rather, was influenced by the
674 C needs of the fungus. It remains to be seen if this observation
675 holds in other ectomycorrhizal host pairings and on larger scales,
676 however, this substantially advances our understanding of the
677 effects of increased C/N availability on ECM fungal function in
678 natural ecosystems and assists in understanding and modelling
679 nutrient transfers in this important interaction.

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Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

KLP, JMP and ICA designed the research. KLP and MW performed experiments. Data was analysed by KLP and VS and curated by VS, VN and IVG. Funding acquisition and project supervision was managed by ICA, JMP, FM, IVG and VN. KLP wrote the manuscript, which was reviewed by all authors.

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Experimental Outcomes	Factors						Number of biological replicates /condition
	Fungal C availability		Plant N availability		Fungal C x Plant N		
	F-ratio	P-value	F-ratio	P-value	F-ratio	P-value	
% Root tips Colonized	2.3786	0.131	12.2034	**0.001**	0.3962	0.533	9-12
Plant C/N ratio	0.4929	0.615	24.466	**1.66E-5**	0.1916	0.827	5-10
N transferred to plant leaves from symbiosis (mg)	12.9987	**0.0048**	2.9597	0.116	0.0011	0.974	3-4
C transferred to fungus via plant (μg)	0.2479	0.626	1.6728	0.217	0.0011	0.974	3-5
% of total N as ^{15}N in mycelium	13.0666	**0.003**	22.7347	**0.0003**	0.1981	0.663	3-5
Fungal C/N ratio	0.0066	0.936	0.2160	0.807	0.2310	0.795	6-10

Table 1 Results from two-way ANOVA examining the effect of fungal C and plant N availability and their interaction (Fungal C x Plant N) on experimental outcomes. Significant P values (<0.05) are represented in bold type.

Figure Legends

Figure 1: Mycorrhizal root tip formation and *E. grandis* N acquisition is affected by the nutrient status of the plant and fungus. (a) Schematic of experimental set up showing the fungal-only nutrient compartment (FC), the plant-only nutrient compartment (PC) and the common symbiosis compartment (SC). (b) Percent lateral root tips colonized in *E. grandis* with (light grey bars) or without (dark grey bars) access to N in contact with *P. albus* with access to either high (10g/L) or low (1.0 g/L) glucose. (c) C to N ratio in the leaves of *E. grandis* with (light grey bars) or without (dark grey bars) access to N grown axenically or in contact with *P. albus* with access to either high (10g/L) or low (1.0 g/L) glucose. (d) Average amount (in μg) of N in the leaves of each *E. grandis* seedling (with (light grey bars) or without (dark grey bars) access to N) acquired from *P. albus* accessing either high (10g/L) or low (1.0 g/L) glucose. +/- SE.

Figure 2: *P. albus* N acquisition is affected by both the nutrient status of the plant and fungus. (a) Estimated average μg C in each *P.albus* colony that is from a plant accessing high N, or a plant without access to N and given either high (10g/L; light grey bars) or low (1.0g/L; dark grey bars) glucose. +/- SE. (b) Percentage of N in *P.albus* that is ^{15}N when grown axenically, with a plant accessing high N, or a plant without access to N and given either high (10g/L; light grey bars) or low (1.0g/L; dark grey bars) glucose. +/- SE. (c) C/N in fungal tissues grown axenically, with a plant accessing high N, or a plant without access to N and given either high (10g/L; light grey bars) or low (1.0g/L; dark grey bars) glucose. +/- SE.

Figure 3: GO term enrichment analysis identifies several enriched biological functions. (a-b) Scatterplot representation of

enriched GO terms corresponding to Biological Process in genes up-regulated (a) or down-regulated (b) in *E. grandis* accessing high plant available N as compared to no plant available N after 48 hours of contact with *P. albus*. Dot sizes correlate to the number of significantly regulated genes in the data set assigned to that GO term and dots are spatially grouped based on GO term similarity. (c-d) Histogram giving the full list of enriched Biological Process GO terms for the data set and the corresponding –log10(p-value) for up-regulated (c) and down-regulated (d) genes. Significantly regulated genes show at least a five-fold change in expression ($p<0.05$; $n=3$). Enrichment analysis and graphical representations were generated using the PlantRegMap and ReviGo online programs respectively.

Supporting Information Legends

Supporting Information Figure S1: Biomass of plant leaves (a), total leaf N content (b), % ^{15}N in plant leaves with unlabeled control plant value indicated by dashed line (c), and percent of total plant leaf N derived from transfer (% NDFT) (d). +/- SE. Results from two-way ANOVAs (unbalanced, Type III) for the effect of carbon (C) or nitrogen (N) availability and their interaction (CxN) on experimental outcomes are indicated (significant results $p<0.05$).

Supporting Information Figure S2: (a) Biomass of fungal control colonies, +/- SE; with significant results (t-test; $p<0.05$) indicated with an asterisk (*). (b) percent of total fungal C obtained via symbiosis, +/- SE. Results from two-way ANOVAs (unbalanced, Type III) for the effect of carbon (C) or nitrogen (N) availability and their interaction (CxN) on experimental outcomes are indicated (significant results $p<0.05$). (c) Average %13C values for growth chamber air and media glucose (black bars) and leaf and fungal tissues generated in the experiment (light grey bars = axenic controls; dark grey = test samples), +/- SE.

Supporting Information Table S1: Composition of media used for the experiment

Supporting Information Table S2: List of differentially regulated genes, log2(fold change) and annotation in *E. grandis* accessing high plant available N, as compared to no plant available N, 48 hours after contact with *P. albus*. Genes shown have at least a five-fold difference in expression (p<0.05; n=3).

Supporting Information Table S3: Complete listing of GO terms significantly enriched in the area of biological process (p<0.01) for significantly differentially regulated genes in the *E. grandis* dataset (p<0.05) when accessing high N, as compared to no N, 48 hours after contact with *P. albus*. Data generated by the online platform PlantRegMap.

Supporting Information Table S4: List of differentially regulated genes, log2(fold change), annotation and signal peptide probability in *P. albus* 48 hours after contact with *E. grandis* accessing high plant available N as compared to no plant available N. Genes shown have at least a two-fold difference in expression (p<0.05; n=3).